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Dnajc3 (HSP40) Modulates Axon Regeneration in the Mouse Optic Nerve

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Abstract

Background: The present study is designed to identify the genes modulating optic nerve regeneration in the mouse. Using the BXD mouse strains as a genetic mapping panel, we examined differential responses to axon regeneration in order to map genomic loci modulating axonal regeneration.

Methods: To study regeneration in the optic nerve, *Pten* was knocked down in the retinal ganglion cells using adeno-associated virus (AAV) delivery of an shRNA, followed by the induction of a mild inflammatory response by an intravitreal injection of Zymosan with CPT-cAMP. The axons of the retinal ganglion cells were damaged by optic nerve crush (ONC). Following a 12-day survival period, regenerating axons were labeled by Cholera Toxin B. Two days later, the regenerating axons within the optic nerve were examined to determine the number of regenerating axons and the distance traveled down the optic nerve. An integral genomic map was made using the regenerative response. Candidate genes were tested by knocking down expression using shRNA or by overexpressing the gene in AAV vectors.

Results: The analysis revealed a considerable amount of differential axonal regeneration across all 33 BXD strains, demonstrated by the number of axons regenerating and the length of the regenerating axons. Some strains (BXD99, BXD90, and BXD29) demonstrated significant axonal regeneration; while other strains (BXD13, BXD18, and BXD34) had very little axon regrowth. Within the regenerative data, there was a 4-fold increase in distance regenerated and a 7.5-fold difference in the number of regenerating axons. These data were used to map a quantitative trait locus modulating axonal regeneration to Chromosome 14 (115 to 119 Mb). Within this locus were 16 annotated genes. Subsequent testing revealed that one candidate gene, *Dnajc3*, modulates axonal regeneration. Knocking down of *Dnajc3* led to a decreased regeneration response in the high regenerative strains (BXD90), while overexpression of *Dnajc3* resulted in an increased regeneration response in C57BL/6J and a low regenerative strain (BXD34).

Conclusion: In this study, *Dnajc3* (encodes Heat Shock Protein 40, HSP40, a molecular chaperone) was identified as a modulator of axon regeneration in mice. This is the first report defining the role of *Dnajc3* (HSP40) in axon regeneration.

Background

The regeneration of mammalian central nervous system axon was once thought to be an

unachievable goal, but it is now becoming a reality. Significant research advances were made in

the regeneration of retinal ganglion cell (RGC) axons through the optic nerve[1-5]. The survival

of RGCs along with the regeneration of axons involve complex interactions of multiple cellular

processes[1, 2, 5-15]. Similarly, the lack of regeneration is linked to a number of different

molecular pathways including transcriptional regulation[2, 16-20] and signaling cascades[1, 21-

23]. Ultimately, these pathways contribute to the survival of the injured RGC, which includes the

modulation of apoptosis[24, 25] and autophagy[1]. There is also a critical change in the response

to growth factors[11, 26-28]. The growth of axons down the optic nerve requires the reactivation

of axon growth programs[29]. These regenerating axons interact with cellular elements that inhibit

axonal growth in the adult Central Nervous System (CNS) that are glial in origin, involving

astrocytes[30, 31], oligodendrocytes [10], or the glial scar[30, 31]. Thus, the regeneration of CNS

axons involves many complex and diverse molecular interactions, making unraveling the overall

regenerative response difficult.

One approach to studying regeneration is to use inbred mouse strains, identifying strains/genetic

backgrounds that facilitate axonal regeneration[8]. Omura et al.[8] tested 9 different inbred strains

and found that one strain (CAST/Ei) was capable of considerable amount of axon regeneration on

inhibitory substrates in tissue culture. The CAST/Ei strain also demonstrated a relatively robust

regeneration in vivo as compared to the C57BL/6 strain. However, using individual inbred mouse

strains makes defining the specific genomic locus responsible for the increased axonal

regeneration difficult. Our group has taken advantage of the BXD genetic reference panel of mice

using a systems biology approach to the study of optic nerve regeneration.

Focusing on a forward-genetics approach, we defined genomic loci modulating the axonal

regeneration produced by knocking down *Pten* (phosphatase and tensin homolog)[1, 3] and

inducing a modest inflammatory response[32] after optic nerve crush (ONC). Looking at the

phenotype of induced axonal regeneration across the BXD recombinant inbred mouse strains, the

genomic elements affecting the response of the retina to optic nerve damage can be defined[33].

MATERIALS AND METHODS

Mice: For the mapping of a locus modulating axonal regeneration, 33 BXD recombinant inbred strains and their parental strains – C57BL/6J and DBA/2J were used in this study. For each strain, a minimum of 4 mice were used. All mice were 60-70 days of age at the time of initial treatment (See Supplemental Table 1). Controls were run with the C57BL/6J (n=6) and DBA/2J (n= 6) mice strains. For testing of candidate genes, the BXD strain with high regenerative responses we chose was BXD90 (n=21), ordered from The Jackson Laboratory. We used this strain to check the regeneration after modulating candidate genes. For testing of the low regenerating mouse strains, we ordered BXD34 mice (n=17) from The Jackson Laboratory. We also examined one parental strain C57BL/6J (n=20), ordered from The Jackson Laboratory. The mice were housed in a pathogen-free facility at Emory University, maintained on a 12-hour light/dark cycle, and provided with food and water ad libitum. All procedures involving animals were approved by the Animal Care and Use Committee of Emory University and were in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Surgery: The optic nerve regeneration protocol developed by others[1, 3, 4] was used to induce regeneration after ONC. The detailed protocol was described in our previous publication[33]. Briefly, the treatment included knocking down of *Pten* and intravitreal injection of Zymosan plus CPT-cAMP. We used AAV-shPTEN-GFP (*Pten* short hairpin RNA-GFP packaged into AAV2 backbone constructs, titer = 1.5×10^{12} vg/ml) to knock down *Pten*. The shRNA target sequence is 5'-AGGTGAAGATATATTCCTCCAA-3' as described by Zukor et al.[34]. The efficient suppression of *Pten* expression in the retinal ganglion cells by this *Pten* shRNA has been proved in a previous study[33]. For all surgeries, the mice were deeply anesthetized with a mixture of 15 mg/kg of xylazine and 100 mg/kg of ketamine. Two weeks prior to ONC, the mice were deeply anesthetized and injected intravitreally with 2µL of AAV-shPTEN-GFP. Optic nerve crush was performed as described by Templeton and Geisert[35]. Briefly, under the binocular operating scope, a small incision was made in the conjunctiva. The optic nerve was visualized and then crushed 1 mm behind the eye with Dumont N7 angled crossover tweezers for 5 seconds, avoiding injury to the ophthalmic artery. Immediately following ONC, Zymosan (Sigma, Z4250, Lot#BCBQ8437V) along with the cAMP analog CPT-cAMP (Sigma, C3912, Lot#SLBH5204V,

total volume 2µL) were injected into the vitreous to induce an inflammatory response and augment

regeneration. The animals were allowed to recover from anesthesia and returned to their cages.

Twelve days after ONC (two days before sacrifice), the animals were deeply anesthetized and

Alexa Fluor® 647-conjugated Cholera Toxin B (CTB, ThermoFisher, Cat.#C34778) was injected

into the vitreous for retrograde labeling of the regenerated axons. All the intravitreal injections and

optic nerve crushes were performed by one well-trained postdoctoral fellow to avoid technical

variation during the surgical procedure. At 14 days after ONC, the mice were deeply anesthetized

and perfused through the heart with phosphate buffered saline (pH 7.3) followed by 4%

paraformaldehyde in phosphate buffer (pH 7.3).

Preparation of the optic nerve: Optic nerves along with optic chiasms and brains were dissected

and post fixed with 4% paraformaldehyde in phosphate buffer overnight. The optic nerve was

cleared with FocusClear™ (CelExplorer, Hsinchu, Taiwan) for up to 4 hours until totally

transparent. A small chamber was built on the slide to provide enough space for the whole nerve

thickness and to keep the nerve from being damaged from flattening. The optic nerve was then

mounted in the chamber using MountClear™ (CelExplorer, Hsinchu, Taiwan) and the slides were

cover-slipped. FocusClear has been used to clear brain tissue for whole brain imaging[36] as well

as clearing of the optic nerve of transgenic zebrafish to observe axon regeneration[37]. It allowed

us to scan the whole thickness of the optic nerve for better understanding of the status of axon

regeneration, while providing clear imaging of regenerated axons from the optical slices scanned

via confocal microscopy for counting. It also allowed us to determine the longest 5 axons or single

axon growth along the nerve from z-stack of the whole nerve.

Quantitation of axon regeneration: Cleared optic nerves were examined on a confocal

microscope by scanning through individual optical slices. Green pseudo-color was used for CTB-

labeled axons in all the optic nerve images of this study for clear visual observation. Stacked

images were taken at 10µm increments, a total of 20-50 optical slices for each optic nerve.

For quantifying the number of axons, we calculated the virtual thickness of an optical slice from

the confocal microscope. As previously described[33], we determined that the thickness of the

optical section was 6µm where refractive index (n) was 1.517, the numerical aperture (Na) was

0.45 and the excitation wavelength was 637nm. Since the optical section was 6µm and the spacing

between optical sections was 10µm, single axons were not counted multiple times.

The number of CTB labeled axons at 0.5 mm from the crush site were counted in at least 6 sections

per case and calculated by the equation ($\Sigma ad = \pi r^2 *$ [average axons/mm]/t) as described by Leon

et al. in 2000[32]. The cross-sectional width of the nerve was measured at the point at which the

counts were taken and was used to calculate the number of axons per millimeter of nerve width.

The total number of axons extending distance d in a nerve having a radius of r, was estimated by

summing over all sections. Since we used confocal images instead of longitudinal cross sections

described in previous studies[3, 4], the optical resolution in z (0.5µm) was considered as the t

(thickness of the slide) in the equation. The number of axons at 0.5mm from the crush site, the

number of axons at 1mm from the crush site, the distance traveled by the 5 longest axons and the

length of the longest single axon were all measured (See Figure 1 of our previous publication[33]).

Interval Mapping

The axon regeneration data was subjected to conventional quantitative trait locus (QTL) analysis

using simple and composite interval mapping using the mm10 assembly. Genotypes were

regressed against each trait using the Haley-Knott equations implemented in the WebQTL module

of GeneNetwork[38, 39]. Empirical significance thresholds of linkage were determined by

permutations [40]. We generated interval maps for all 4 regeneration measures: number of axons

at 0.5mm from the crush site, number of axons at 1mm from the crush site, distance traveled by

the 5 longest axons and the length of the longest single axon. In the present paper, we made a

synthetic trait for axon regeneration by combining all 4 datasets into a single measure and used

this to produce an interval map. To identify loci, and also to nominate candidate genes, we used

the following approaches: interval mapping for the traditional phenotypes, candidate gene

selection within the QTL region, cis-eQTL analysis of gene expression, and trans-eQTL analysis.

AAV vector

To knock down the genes of interest, we used a similar AAV vector to the *Pten* knock down as

previously described[33]. Briefly, short hairpin RNA was designed for genes of interest, including

Dnajc3 (NM_008929.3) and *Uggt2* (NM_001081252.2), using the shRNA-designer from Biosettia

(https://biosettia.com/support/shrna-designer/). The targeting sequence was selected on the exon

region that contained no single nucleotide polymorphisms (SNPs) between C57BL/6J and DBA/2J

strains. The shRNA sequence for the gene of interest was inserted into the AAV-shPTEN-GFP

replacing the shRNA sequence for *Pten*. The transfection efficiency for these vectors is the same

as AAV-shPTEN-GFP, which was tested previously[33]. For AAV-shDnajc3-GFP, the shRNA

target sequence is 5'-GCAACCAGCAAATATGAAT-3', virus titer = 1.6×10^{14} vg/ml. For AAV-

shUggt2-GFP, the shRNA target sequence is 5'-GCCTGGGATTATCAGCAAT-3', virus titer =

1.4×10^{14} vg/ml. For the overexpression of *Dnajc3* in the RGCs, the mRNA sequence of *Dnajc3*

(NM_008929.4) was packed into the AAV vector. The virus titer was 4×10^{12}. All of our plasmids

were made at Emory Integrated Genomic Core (https://cores.emory.edu/eigc/) and were packed

into AAV2 at Emory Viral Vector Core (https://neurology.emory.edu/ENNCF/viral_vector/).

Previously[33], we have demonstrated that the AAV transduces approximately 54% of the retinal

ganglion cells in mice.

Testing Candidate Genes by Knockdown or Overexpression.

Candidate genes were tested in our model optic nerve regeneration system. The AAV vector either

with shRNA for knocking down a gene or with full-length sequence for overexpressing a gene

were injected into the vitreous chamber one week before the start of the regeneration protocol. The

vectors included: AAV-sh*Uggt2*-GFP and AAV-sh*Dnajc3*-GFP for knock down, or AAV-*Dnajc*3

for overexpression. For testing the effects of knocking down candidate genes, we used a BXD

strain that demonstrated robust regeneration, BXD90. The BXD90 mice received an intravitreal

injection of either AAV-sh*Uggt2*-GFP or AAV-sh*Dnajc3*-GFP. For testing the effects of

overexpressing a gene, we used either a low regenerating strain, BXD34, or the parental strain,

C57BL/6J. The AAV-*Dnajc3* vector was injected into the intravitreal chamber one week before

the initiation of the regeneration protocol. The same strain of mice receiving AAV-GFP were used

as a control group for both the knockdown and overexpression experiments.

Statistical Analysis: Data are presented as Mean ± SE (Standard Error of the Mean).
Differences in axon counts and regeneration distance between two strains were analyzed by
Exact Wilcoxon-Mann-Whitney Test Calculator[41] (https://ccb-compute2.cs.uni-
saarland.de/wtest/?id=www/www-ccb/html/wtest,). A value of $p < 0.05$ was considered
statistically significant.

Results

Axon regeneration was examined in 33 strains of mice (31 BXD strains and the two parental
strains, C57BL/6J and DBA/2J). Of these 33 strains, the data from nine were used in a previous
study demonstrating that optic nerve regeneration is a complex trait[42]. The BXD strain set has
proven to be a valuable genetic reference panel in vision research[43]. Here we examine the extent
of axon regeneration 14 days following optic nerve crush. There was a surprising difference in
regenerative capacity that was dependent upon the specific BXD strain examined (Figure 1). Some
strains (BXD13, BXD18, and BXD34) showed very little axon regeneration; while other strains
(BXD99, BXD90, and BXD29) displayed a significant amount of axon regrowth down the optic
nerve (Figure 1). In all cases, optic nerve crush followed by the axon regeneration treatment led to
more axonal regrowth than observed in the two control cases (C57BL6J and DBA/2J) which
received optic nerve crush only (Figure 1). The axon regeneration for each strain was quantified
by counting the number of regenerating axons at 0.5mm (Figure 2A) and 1mm (Figure 2B) from
the crush site (See Supplemental Table 1). The distance the axons had traveled down the optic
nerve was also quantified measuring the length of the longest 5 axons (Figure 2C) and the distance
traveled by the longest single axon (Figure 2D). These data were compared to axon regeneration
in control mice that did not receive the regeneration treatment (Figure 2). When examining the
regenerative capacity of the different BXD strains, the number of regenerating axons at 0.5mm
varies by 7.5-fold, with the lowest number in strain BXD34 being an average of 135.4 axons and
the greatest number of regenerating axons in strain BXD90 being an average of 1030 axons (Figure
2A). A similar pattern is observed 1mm from the crush site, with the lowest average number of
regenerating axons in BXD18 and the highest average number of regenerating axons in BXD90
(Figure 2B). When examining the distance axons have regenerated down the optic nerve, the

general pattern is similar to that observed for the number of regenerating axons. The fold change

in axon growth for the longest 5 regenerating axons was 4 with the shortest axons in strain BXD34

and the longest axons in BXD29 (Figure 2 C). This is also the case for the longest single axon with

the shortest strain being BXD34 and the longest being BXD29 (Figure 2D). The data suggest that

the number of regenerating axons and the distance the axons travel down the optic nerve reflect a

similar genetic underpinning for the strains with the fewest axons also have the shorter

regenerating axons. It is also the case that the strains with the greatest number of axons also have

axons that travel the greatest distance down the optic nerve.

Interval Mapping

These data were used to map genomic loci that may modulate the regenerative response in the

BXD strains. The unbiased, forward genetics approach was used to create a genome-wide interval

map for each of the measures of regeneration: number of axons 0.5mm from the crush site, number

of axons 1mm from the crush site, distance traveled by the 5 longest axons and the length of the

longest single axon (Supplemental Figure 1). All four interval maps showed a similar pattern with

a large peak being on distal Chromosome (Chr) 14. The peak on Chr 14 (115 Mb to 119 Mb) is

above the suggestive level for all measures. The map on the longest single axon has a QTL that

reached the significance level (Supplemental Figure 1D). Since all of the genome wide scans were

similar, all of these data were combined to create a synthetic trait, "axonal regeneration". The

genome-wide scan for axonal regeneration revealed the same significant peak on Chr 14 (Figure

3). For further analysis, we used data from the combined synthetic trait, axon regeneration, to

identify the QTL modulating axon regeneration as well as defining the candidate genes within the

region.

Candidate Genes

The interval map for the longest single axon and the synthetic trait axon regeneration both had a

significant QTL peak on Chr 14. To identify genes modulating axonal regeneration in the BXD

strains, we examined a 4 Mb region around the peak ranging from 115 Mb to 119 Mb (Figure 3).

Within this region, there were 16 annotated genes: *Mir18*, *Mir19a*, *Mir20a*, *MIr17hg*, *Gpc5*, *Gpc6*,

Gm19845, *Dct*, *Tgds*, *Gpr180*, *Sox21*, *Abcc4*, *Cldn10*, *Dzip1*, *Dnajc3* and *Uggt2*. In general, ideal

candidate genes should have a nonsynonymous SNP between the two parental strains that affects

protein sequence and function, or there should be genomic elements with cis-QTLs affecting

expression levels. Of the 16 genes within the region, two had nonsynonymous SNP: *Abcc4* and

Cldn10. To determine if any of these amino acid changes would affect protein function, we ran a

SIFT analysis[44]. All of the changes in both genes were tolerated and should not disrupt protein

function. Thus, these two genes did not appear to be good candidates and were removed as

potential candidate genes. Two genes were found with cis-QTLs: *Dnajc3* and *Uggt2*. The

difference in expression levels of these two candidate genes was confirmed by examining RNAseq

comparisons between the parental strains[45]. Furthermore, both of these genes are expressed in

most of the retinal ganglion cell types in single cell RNAseq datasets[46]. These data indicate that

both *Dnajc3* and *Uggt2* are good candidate genes for modulating axonal regeneration.

Knockdown of *Dnajc3* and *Uggt2*

To further investigate the roles of the two candidate genes in optic nerve regeneration, we extended

our study using the existing regeneration model. As an initial test, we chose one of the high

regenerative strains, BXD90, to examine the effects of knocking down gene expression using AAV

delivery of shRNA. AAV-shRNA directed against either *Uggt2* or *Dnajc3* was injected into the

vitreous chamber of the mouse eye, knocking down the expression of each candidate gene. The

injection was given a week prior to *Pten* knockdown (Figure 4A). This allowed the effective

transduction of cells with AAV carrying candidate gene-specific shRNA. This also produced a

decrease in gene expression prior to the beginning of the experiment without disrupting the

established neural regeneration experimental protocol. The control group received AAV-GFP at

the same time point. A successful knockdown of the targeting gene *Dnajc3* is demonstrated in

Figure 4 (B-D).

Our results showed that selectively knocking down *Uggt2* in RGCs did not impact optic nerve

regeneration (Figure 5 A-D), making it unlikely to be the gene modulates axon regeneration in the

BXD strains. Knocking down of *Dnajc3* in RGCs decreased the number of axons regenerating in

the optic nerve (Figure 5A and 5B) as well as the distance that the axons traveled down the optic

nerve (Figure 5C and 5D) after regeneration treatment for ONC. Quantifying the regenerative

capacity revealed a significant 24.6% decrease (767.6 ± 44.5 vs 1017.9 ± 46.5, $p < 0.05$) in the

number of axons at 0.5mm from the injury site compared with the GFP group. There was also a

significant 53.7% decrease (8±1 vs 17.3±1.6, $p < 0.05$) at 1mm from the crush site. A similar result

was observed in the distance axons regenerated down the optic nerve. There was a 28.5% decrease

in the distance of the 5 longest axons in the *Dnajc3* knockdown mice relative to the GFP controls

(1±0.1 vs 1.4±0.1, $p < 0.05$), and for the longest single axon there was a 38.5% decrease in distance

traveled (1.3±0.1 vs 2±0.2, $p < 0.05$). Detailed data is shown in Supplementary table 2. These data

indicate that *Dnajc3* is a good candidate gene for modulating axon regeneration down the optic

nerve.

The axonal regeneration observed following the knockdown of *Dnajc3* in BXD90, a high

regenerative strain, is significantly greater than that observed in BXD18, a low regenerative strain

(Figure 5). This is the case for the number of regenerating axons as well as the distance the axons

travel down the optic nerve. Although the regenerative capacity is significantly diminished in

Dnajc3 knock down in BXD90 mice relative to the GPF controls ($p < 0.05$ Mann-Whitney U test),

it still does not reach the level of the low regenerative strain, BXD18 (Figure 5). These findings

indicate that *Dnajc3* modulates optic nerve regeneration in the BXD strain set; however, *Dnajc3*

is not solely responsible for the variation in regeneration observed in the BXD strain set, for the

increased regeneration is not completely blocked by knocking down *Dnajc3*.

Overexpression of *Dnajc3*:

To provide additional evidence that *Dnajc3* modulates axonal regeneration, we used AAV to

overexpress *Dnajc3* in the RGCs of one of the parental strains (C57BL/6J) and one of the lowest

regenerating strains (BXD34). The full cDNA sequence of *Dnajc3* was placed into a AAV vector

and packaged into AAV2 capsid. The AAV-*Dnajc3* was injected into the vitreous of 10 C57BL/6J

mice and 9 BXD34 mice, while the controls were injected with AAV-GFP in 10 C57BL/6J mice

and in 8 BXD34 mice one week prior to the optic nerve regeneration protocol (similar schedule as

shown in Figure 4A). In both C57BL/6J and BXD34 strains, there was a significant increase

($p<0.05$ Mann-Whitney U test) in the number of regenerating axons (Figure 6 A and B) and the

distance the regenerating axons traveled down the nerve (Figure 6 C and D). Detailed data is shown

in Supplementary table 2. Thus, overexpressing *Dnajc3* in low regenerating strains increased the

amount of axon regeneration down the optic nerve. Taken together, the decrease in axonal

regeneration following knocking down *Dnajc3* and the increase in regeneration following
overexpressing *Dnajc3*, demonstrates that one of the genomic elements modulating axonal
regeneration in the BXD strain set is *Dnajc3*.

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DISCUSSION
In the present study, we have used a forward-genetics approach to identify a genomic locus that
modulates the regenerative response induced by knocking down *Pten* and creating a mild
inflammatory response with Zymosan and CPT-cAMP. This approach identified one locus on
Chromosome 14 (115 Mb to 119 Mb) that modulates optic nerve regeneration. Within this locus,
there were 16 annotated genes, two of which were good candidate genes (*Uggt2* and *Dnajc3*).
Subsequent testing eliminated *Uggt2* as a modulator of axonal regeneration and demonstrated that
changes in expression levels of *Dnajc3* can alter the regenerative response. Knocking down
Dnajc3 in a high regenerating strain of mice decreases axon regeneration, while overexpressing
Dnajc3 in a low regenerating strain increases the ability of axons to regenerate in the optic nerve.
In the BXD strain set, *Dnajc3* modulates the regenerative response produced by knocking down
Pten and inducing a mild inflammatory response with Zymosan and CPT-cAMP.

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Dnajc3 is expressed in RGCs at relatively high levels and is localized to the endoplasmic reticulum
(ER)[47]. *Dnajc3* encodes p58IPK, also known as heat shock protein 40 (HSP40)[48, 49]. Like
many heat shock proteins, HSP40 is an ER chaperone playing a role in the unfolded protein
response (UPR) when neurons are stressed[48, 49]. The UPR plays an essential role in maintaining
cellular homeostasis in CNS neurons by activating in stress conditions when there's an
accumulation of misfolded or unfolded proteins in the ER. Unlike many of the chaperone proteins,
HSP40 can also function as an inhibitor of eIF2a, a protein kinase localized to the inner surface of
the endoplasmic reticulum[49]. Crucially, in the context of retinal health, the role of HSP40
(p58IPK) in protein homeostasis and anti-inflammatory response has been identified as protective
against retinal ganglion cell degeneration[47, 50, 51], a primary cause of visual impairment in
conditions like glaucoma. The protective effect of HSP40 was revealed by Zhang's group from
both in vitro[47, 51] and in vivo[50, 51] experiments. They reported that deficiency of HSP40

makes the RGCs more susceptible to cell death by the ER stress inducer; while overexpression of
p58IPK by AAV increases RGC survival under ER stress[51]. HSP40 protects from retinal
ischemia/reperfusion and from elevated intraocular pressure (IOP) caused by microbead injections
into the anterior chamber[51]. Knocking down HSP40 in vivo and in vitro causes a decrease in
RGC survival while overexpressing the protein promotes neuronal survival in culture[51]. These
data strongly support the role of HSP40 in protecting RGCs from stress induced cell death.

Using the BXD mouse strains, we show that *Dnajc3* is a genomic element modulating axonal
regeneration. This includes the number of regenerating axons at 0.5mm and 1mm from the crush
site, as well as the distance these axons travel down the optic nerve, whether it is the 5 longest
axons or the single longest axon. Our results suggest a potential mechanism responsible for the
partial increase in the number of regenerating we observed in the present study. Previous
studies[47, 50] demonstrate that overexpressing *Dnajc3* (HSP40) is neuroprotective for RGCs in
an elevated IOP model of glaucoma and in an ischemia/reperfusion model in the mouse. If *Dnajc3*
can act as a neuroprotective genomic element, then the increase in the number of regenerating
axons could be directly due to the fact that more RGCs are surviving the insult to the optic nerve.
Thus, the number of regenerating axons may be directly related to the neuroprotective effects of
Dnajc3 in RGCs; the more RGCs that survive following optic nerve crush, the more axons that are
available to regenerate down the optic nerve.

Interestingly, we have found that the genomic loci modulating the distance axons regenerate down
the optic nerve are similar to those affecting the number of regenerating axons. Clearly, RGC
survival is one of the fundamental aspects necessary for axon regeneration, for if the cell body
dies, the cell will not be able to regrow down the optic nerve. That being said, there is increasing
evidence that RGC survival and axon regeneration can be two completely independent processes.
An example of this is the effect of *Sox11* on retinal injury. The overexpression of *Sox11* in mouse
RGCs promotes regeneration[5]. Interestingly, it does not promote regeneration in some of the
RGC subtypes. αRGCs, which are normally associated with induced regeneration down the optic
nerve, are killed by *Sox11* overexpression[5]. Thus, the increased axonal regeneration induced by
Sox11 overexpression must be due to a response from a different RGC subtype, not the αRGCs.
The downregulation of *Sox11* increased RGC survival following injury of optic nerve axons[52,

53]. Even though there was an increase in RGC survival, there was not an improved regeneration

of axons down the optic nerve in an induced optic regeneration model[52, 53]. Thus, the

neuroprotection of RGCs does not result in an obligatory increase in axonal regeneration. That

being said, there may be elements that facilitate RGC survival and facilitate axonal regeneration.

Independent of RGC survival, HSP40 (p58IPK) might also influence axon regeneration in the

following aspects. In the past, it was believed that while neurons in the peripheral nervous system

(PNS) could regenerate after injury, mature neurons in the CNS could not. However, numerous

subsequent studies have achieved regeneration of CNS neurons through experimental methods. In

the injured PNS, regeneration involves the local synthesis of damage signals and translation of

growth-promoting mRNA, such as Gap-43 and β-actin[54]. Under certain conditions, similar

mRNA was also detected in injured CNS axons[55]. Local protein synthesis events detected in

embryonic axons and adult PNS axons play a pivotal role in neuronal regeneration[56]. For CNS

neurons, axon regeneration requires the fulfillment of several conditions. One critical aspect is the

synthesis and transportation of proteins to supply the nutrients and raw materials necessary for

axon regeneration. First, protein synthesis is vital for the formation of growth cones. Research has

shown that inhibiting protein synthesis impairs growth cones, which are essential structures for

axon regeneration[57]. Second, processes involved in axon regeneration, such as retrograde

signaling, growth cone formation, and axon elongation, all depend on local translation[56]. Adult

local transcriptomes detected in retinal ganglion cells, as well as translation mechanisms in several

adult brain regions, have confirmed local translation in mature CNS axons[58-60]. Moreover,

during CNS development, ribosome localization in axons might gradually decrease, indicating that

ribosome positioning and mRNA translation specificity change with maturity[58, 61]. After injury,

the local translation dynamics of adult CNS axons might also decline, such as reduced axonal

transcripts or a lack of functional ribosomes[62]. Furthermore, local translation might be hindered

due to incorrect mRNA transport or the retention of mRNA in stress granules[54, 63]. Therefore,

it can be inferred that the limited regenerative capacity of CNS axons might be related to reduced

local translation. Supporting this, earlier research by Park and colleagues[1] demonstrated that

enhancing protein synthesis by augmenting mTOR signaling activity through silencing the *Pten*

gene greatly promotes optic nerve axon regeneration, possibly by influencing local axonal

translation[1]. In this experiment, the establishment of the axon regeneration model was based on

429 silencing the *Pten* gene. As a chaperone protein in ER stress, HSP40 may alleviate ER stress,

430 thereby enhancing the protein synthesis capability of the ER as well as the protein translation and

431 transportation abilities at the site of axon injury. This series of effects means that neurons

432 influenced by it not only have enhanced survival capabilities but also exhibit significantly

433 increased axonal regenerative abilities.

436 ***Dnajc3* is not the only modulator of axon regeneration in the BXD strains:**

437 When we examine the effects of altering the expression of *Dnajc3*, it is clear that it is not the sole

438 element affecting the difference in axonal regeneration across the BXD strain examined in the

439 current study. Knocking down *Dnajc3* in a strain with extensive axonal regeneration does not

440 reduce the axonal regrowth to the level of the lower expressing strains. Furthermore,

441 overexpressing *Dnajc3* in a strain with limited axonal regrowth does not enhance the regeneration

442 to the same level as a strain with robust optic nerve regeneration. Taken together, these data

443 indicate that there is at least a second genomic element interacting with *Dnajc3* or directly

444 modulating optic nerve regeneration that differs between the two strains of mice.

446 **Conclusion:**

447 Optic nerve regeneration is a highly complex process with many factors interacting and influencing

448 each other. Using a forward-genetics approach, we identified *Dnajc3* as a genomic element

449 modulating axon regeneration in the mouse optic nerve. The protein it encodes, HSP40, is one

450 element modulating axonal regeneration in the mouse. The gene product of HSP40 is an important

451 factor within the retinal ganglion cells, and the protein either overexpressing or knocking down its

452 expression affects the regenerative capacity of injured axons in an experimentally induced optic

453 nerve regeneration model. One of the underlying mechanisms in this increased regeneration is

454 probably due to its role in protecting RGCs from death. In addition, it may improve the ability of

455 neurons to overcome the unfolded protein response that complicates axons regrowth.

456 **List of Abbreviations:** Adeno Associated Virus (AAV), Optic Nerve Crush (ONC), Retinal

457 Ganglion Cell (RGC), Heat Shock Protein 40 (HSP40), Quantitative Trait Locus (QTL), Cholera

458 Toxin B (CTB), Chromosome (Chr), Unfolded Protein Response (UPR) Central Nervous System

(CNS), Peripheral Nervous System (PNS), Endoplasmic Reticulum (ER), Intraocular Pressure
(IOP).

Declarations:

Ethics Approval, All procedures involving animals were approved by the Animal Care and Use
Committee of Emory University and were in accordance with the ARVO Statement for the Use of
Animals in Ophthalmic and Vision Research.

Consent to Publish, Not Applicable

Availability of Data and Materials, The data quantifying axonal regeneration in the BXD mouse
strain set is available on GeneNetwork (genenetwork.org): number of axons at 0.5mm
(BXD_27559), number of axons at 1mm (BXD_27560), the distance traveled by the 5 longest
axons (BXD_27561) and the length of the single longest axon (BXD_27562). All vectors used in
this study are available upon request.

Competing Interests, The authors declare that they have no competing interests.

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493
Figure 1. The figure is a series of photomicrographs from 17 optic nerves selected from 15 different
strains of mice (N ≧ 4 in each strain). The two images to the left are from control mice with optic
nerve crush only (Control C57BL/6J and Control DBA/2J). The remaining nerves are from animals
in which the optic nerve was crushed and regeneration treatments were applied. Red asterisks
represent the crush site. The strains from left to right are: C57BL/6J crush only, DBA/2J crush
only, BXD34, BXD13, BXD18, C57BL/6J, BXD85, BXD40, BXD38, BXD75, DBA/2J, BXD28,
BXD31, BXD73, BXD99, BXD90 and BXD29. The scale to the left represents 200μm.
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Figure 2. The number of axons at 0.5mm from the crush site (A), the number of axons 1mm from
the crush site (B) are shown. In addition, the distance traveled by the longest 5 regenerating axons
(C) and the longest single axon (D) is illustrated, in two control strains (C57BL/6J and DBA/2J
untreated mice) and in 33 strains that went through the regeneration protocol. Each animal is
represented by a single dot, with the pink dots for females and the light blue dots for males.
Comparing the strains with the greatest regeneration with the strains with the least regeneration,
there is a 7.5-fold difference in the number of axons at 0.5mm from crush site (A) and 38-fold
difference at 1mm from crush site (B). There is an approximate 4-fold difference in the distance
of the longest 5 axons and 3-fold difference in the distance of the longest single axon, when
comparing the strains with longest axons to the ones with the shortest axons. The fact that the
parental strains are not the extremes of the distributions indicates that there is genetic transgression
in axonal regeneration, suggesting a minimum two genomic loci modulating the phenotype of axon
regeneration.
515
Figure 3. A genome-wide interval map of axon regeneration (A). The interval map plots the
likelihood ratio statistic (LRS) across the genome from chromosome 1 to chromosome X. The
light gray line is the suggestive level and the light red line is the genome-wide significance ($P =$
0.05). When the optic nerve regeneration measures were mapped to the mouse genome, there was
a significant association between optic nerve regeneration and a locus on Chromosome 14. A map

of gene locations across Chr 14 from 113 to 123Mb is shown (D). The locations of genes within

this region are schematically shown in the top panel (B). The haplotype map for the 33 strains in

the optic nerve regeneration dataset is illustrated in the middle panel with each strain indicated on

the right (C). Red represents the B6 alleles, green defines the D2 alleles, blue represents regions

of the DNA that are heterozygotic and gray is unmapped. The genomic markers used in the

mapping process are listed at the bottom of the haplotype map. At the far right is a list of the

specific BXD RI strains and the associated regeneration measurements going from the most

regeneration at the top to the least at the bottom. A QTL map of regeneration on Chr 14 is shown

below the haplotype map. An LRS above the pink line is statistically significant ($p<0.05$). A

positive additive coefficient (green line) indicates that D2 alleles are associated with higher trait

values. The significant peak for optic nerve regeneration is from 114 to 119 Mb. Vertical orange

lines at the bottom of the plot show the SNPs on Chr 14.

Figure 4. The modified regeneratin protocol is illustrated in A, 7 days prior to the knockdown of

PTEN (Day 0), AAV-*Dnajc3* KD or AAV-GFP (control) was injected into the eye. At day 7,

AAV-sh*Pten*-GFP was then injected into the eye. Two weeks later (Day 21), the optic nerve is

crushed and Zymosan and cAMP were injected into the eye. On day 33, the eye was injected with

Alexa Fluor® 647-conjugated CTB and two days later the animal was perfused. In panels B-D,

flatmount of a retina injected with AAV-sh*Dnajc3*-GFP are shown. In the first panel B, the staining

for GFP is shown labeling RGCs. Panel C shows the labeling for HSP40 (*Dnajc3*). Notice the lack

of HSP40 staining in the GFP expressing RGCs (white arrows in B-D). The final panel D is a

merged image of panels B and C. The scale bar in D represents 50µm.

Figure 5. *Uggt2* and *Dnajc3* knockdown in BXD90. BXD90 was one of the strains with robust

optic nerve regeneration induced by our regeneration protocol. We used AAV-shRNAs to

knockdown the two candidate genes, *Uggt2* and *Dnajc3*, in RGCs prior to testing for optic nerve

regeneration. Each animal is represented by a dot and the means are presented as blue diamonds.

In panel A, the number of axons at 0.5mm from the crush site is shown. The first column is the

data from control mice receiving AAV-GFP. The effects of knocking down *Uggt2* is shown in the

next column (BXD90_*Uggt2*_KD). The data from knocking down *Dnajc3* is shown next

(BXD90_*Dnajc3* KD). Notice a significant decrease in the number of axons regenerating ($p < 0.05$

Mann-Whitney U test). The data from one of the low regenerating strains (BXD18) is also shown

for comparison. Similar results are shown for number of axons at 1mm from the crush site (B).

This is also the case for the distance axons regrew down the optic nerve, for both the 5 longest

axons (C) and the longest single axon (D). These data demonstrate that knocking down *Uggt2* does

not affect axon growth in BXD90. It also demonstrates that knocking down *Dnajc3* can modulate

the amount of axon regeneration. Thus, *Dnajc3* is the best candidate at the Chr 14 locus for

modulating axonal regeneration.

Figure 6. Overexpression of *Dnajc3* in C57BL/6J mice and in BXD34 mice. BXD34 was one of

the strains with least optic nerve regeneration induced by our regeneration protocol. *Dnajc3* was

over expressed using AAV-*Dnajc3* to express it in RGCs prior to testing for optic nerve

regeneration. Each animal is represented by a dot and the means are presented as blue diamonds.

In panel A, the number of axons at 0.5mm from the crush site is shown. The first column is the

data from control C57BL/6J mice receiving AAV-GFP (B6_GFP). The effects of overexpressing

Dnajc3 are shown in the next column (B6_*Dnajc3*_OE). There was a significant (p < 0.05 Mann-

Whitney U test) increase in the number of axons when *Dnajc3* was overexpressed. The next

column shows the data from the BXD34 GFP control (BXD34_GFP). The data from

overexpressing *Dnajc3* in BXD34 is the last column (BXD34_*Dnajc3*_OE). Notice a significant

increase in the number of axons regenerating (p < 0.05 Mann-Whitney U test). Similar results are

shown for number of axons at 1mm from the crush site (B). This is also the case for the distance

axons regrew down the optic nerve, for both the 5 longest axons (C) and the longest single axon

(D). These data demonstrate that overexpressing *Dnajc3* in a low regenerating strain of either

C57BL/6J or BXD34 increases the regeneration of axons following optic nerve crush.

Supplemental Figure 1. Genome-wide interval maps for the number of axons at 0.5mm from the

crush site (A) and the number of axons 1mm from the crush site (B) are shown. In addition, the

distance traveled by the longest 5 regenerating axons (C) and the longest single axon (D) are

illustrated. The interval map plots the likelihood ratio statistic (LRS) across the genome from

chromosome 1 to chromosome X. The light gray line is the suggestive level and the light red line

is the genome-wide significance (*p*=0.05). When the optic nerve regeneration measures were

mapped to the mouse genome, a significant association between regeneration and a locus on

Chromosome 14 was observed.

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775 Figures
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Figure 1. The figure is a series of photomicrographs from 17 optic nerves selected from 15
different strains of mice (N ≧ 4 in each strain). The two images to the left are from control mice
with optic nerve crush only (Control C57BL/6J and Control DBA/2J). The remaining nerves are
from animals in which the optic nerve was crushed and regeneration treatments were applied.
Red asterisks represent the crush site. The strains from left to right are: C57BL/6J crush only,
DBA/2J crush only, BXD34, BXD13, BXD18, C57BL/6J, BXD85, BXD40, BXD38, BXD75,
DBA/2J, BXD28, BXD31, BXD73, BXD99, BXD90 and BXD29. The scale to the left
represents 200µm.
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Figure 2. The number of axons at 0.5mm from the crush site (A), the number of axons 1mm from
the crush site (B) are shown. In addition, the distance traveled by the longest 5 regenerating
axons (C) and the longest single axon (D) is illustrated, in two control strains (C57BL/6J and
DBA/2J untreated mice) and in 33 strains that went through the regeneration protocol. Each
animal is represented by a single dot, with the pink dots for females and the light blue dots for
males. Comparing the strains with the greatest regeneration with the strains with the least
regeneration, there is a 7.5-fold difference in the number of axons at 0.5mm from crush site (A)
and 38-fold difference at 1mm from crush site (B). There is an approximate 4-fold difference in
the distance of the longest 5 axons and 3-fold difference in the distance of the longest single
axon, when comparing the strains with longest axons to the ones with the shortest axons. The
fact that the parental strains are not the extremes of the distributions indicates that there is
genetic transgression in axonal regeneration, suggesting a minimum two genomic loci
modulating the phenotype of axon regeneration.
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Figure 3. A genome-wide interval map of axon regeneration (A). The interval map plots the
likelihood ratio statistic (LRS) across the genome from chromosome 1 to chromosome X. The light
gray line is the suggestive level and the light red line is the genome-wide significance ($P = 0.05$).
When the optic nerve regeneration measures were mapped to the mouse genome, there was a
significant association between optic nerve regeneration and a locus on Chromosome 14. A map of
gene locations across Chr 14 from 113 to 123Mb is shown (D). The locations of genes within
this region are schematically shown in the top panel (B). The haplotype map for the 33 strains in
the optic nerve regeneration dataset is illustrated in the middle panel with each strain indicated
on the right (C). Red represents the B6 alleles, green defines the D2 alleles, blue represents
regions of the DNA that are heterozygotic and gray is unmapped. The genomic markers used in
the mapping process are listed at the bottom of the haplotype map. At the far right is a list of the
specific BXD RI strains and the associated regeneration measurements going from the most

regeneration at the top to the least at the bottom. A QTL map of regeneration on Chr 14 is shown
below the haplotype map. An LRS above the pink line is statistically significant (p<0.05). A
positive additive coefficient (green line) indicates that D2 alleles are associated with higher trait
values. The significant peak for optic nerve regeneration is from 114 to 119 Mb. Vertical orange
lines at the bottom of the plot show the SNPs on Chr 14.

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Figure 4. The modified regeneratin protocol is illustrated in A, 7 days prior to the knockdown of
PTEN (Day 0), AAV-*Dnajc3* KD or AAV-GFP (control) was injected into the eye. At day 7,
AAV-sh*Pten*-GFP was then injected into the eye. Two weeks later (Day 21), the optic nerve is
crushed and Zymosan and cAMP were injected into the eye. On day 33, the eye was injected
with Alexa Fluor® 647-conjugated CTB and two days later the animal was perfused. In panels
B-D, flatmount of a retina injected with AAV-sh*Dnajc3*-GFP are shown. In the first panel B, the
staining for GFP is shown labeling RGCs. Panel C shows the labeling for HSP40 (*Dnajc3*).
Notice the lack of HSP40 staining in the GFP expressing RGCs (white arrows in B-D). The final
panel D is a merged image of panels B and C. The scale bar in D represents 50µm.
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Figure 5. *Uggt2* and *Dnajc3* knockdown in BXD90. BXD90 was one of the strains with robust
optic nerve regeneration induced by our regeneration protocol. We used AAV-shRNAs to
knockdown the two candidate genes, *Uggt2* and *Dnajc3*, in RGCs prior to testing for optic nerve
regeneration. Each animal is represented by a dot and the means are presented as blue diamonds.
In panel A, the number of axons at 0.5mm from the crush site is shown. The first column is the
data from control mice receiving AAV-GFP. The effects of knocking down *Uggt2* is shown in
the next column (BXD90_*Uggt2*_KD). The data from knocking down *Dnajc3* is shown next
(BXD90_*Dnajc3* KD). Notice a significant decrease in the number of axons regenerating (p <
0.05 Mann-Whitney U test). The data from one of the low regenerating strains (BXD18) is also
shown for comparison. Similar results are shown for number of axons at 1mm from the crush site
(B). This is also the case for the distance axons regrew down the optic nerve, for both the 5
longest axons (C) and the longest single axon (D). These data demonstrate that knocking down
Uggt2 does not affect axon growth in BXD90. It also demonstrates that knocking down *Dnajc3*
can modulate the amount of axon regeneration. Thus, *Dnajc3* is the best candidate at the Chr 14
locus for modulating axonal regeneration.
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Figure 6. Overexpression of *Dnajc3* in C57BL/6J mice and in BXD34 mice. BXD34 was one of
the strains with least optic nerve regeneration induced by our regeneration protocol. *Dnajc3* was
over expressed using AAV-*Dnajc3* to express it in RGCs prior to testing for optic nerve
regeneration. Each animal is represented by a dot and the means are presented as blue diamonds.
In panel A, the number of axons at 0.5mm from the crush site is shown. The first column is the
data from control C57BL/6J mice receiving AAV-GFP (B6_GFP). The effects of overexpressing
Dnajc3 are shown in the next column (B6_*Dnajc3*_OE). There was a significant (p < 0.05 Mann-
Whitney U test) increase in the number of axons when *Dnajc3* was overexpressed. The next
column shows the data from the BXD34 GFP control (BXD34_GFP). The data from
overexpressing *Dnajc3* in BXD34 is the last column (BXD34_*Dnajc3*_OE). Notice a significant
increase in the number of axons regenerating (p < 0.05 Mann-Whitney U test). Similar results are
shown for number of axons at 1mm from the crush site (B). This is also the case for the distance
axons regrew down the optic nerve, for both the 5 longest axons (C) and the longest single axon
(D). These data demonstrate that overexpressing *Dnajc3* in a low regenerating strain of either
C57BL/6J or BXD34 increases the regeneration of axons following optic nerve crush.
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Supplemental Figure 1. Genome-wide interval maps for the number of axons at 0.5mm from the
crush site (A) and the number of axons 1mm from the crush site (B) are shown. In addition, the
distance traveled by the longest 5 regenerating axons (C) and the longest single axon (D) are
illustrated. The interval map plots the likelihood ratio statistic (LRS) across the genome from
chromosome 1 to chromosome X. The light gray line is the suggestive level and the light red line is
the genome-wide significance ($p = 0.05$). When the optic nerve regeneration measures were mapped
to the mouse genome, a significant association between regeneration and a locus on Chromosome 14
was observed.
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